REMARKS

The Invention

In general, the invention features (i) polypeptides that consist of a BIR3 domain from human or mouse XIAP, HIAP-1, or HIAP-2, (ii) polypeptides having 95% identity to one of these BIR3 domains, and (iii) polypeptides that include one of these BIR3 domains. Polypeptides of the invention are capable of inhibiting apoptosis of a mammalian cell when the polypeptide is expressed therein.

The Office Action

Claims 1-21 are pending. All claims stand rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-6 of U.S. Patent No. 6,656,704. All claims stand further rejected as lacking enablement. Claims 1-7 and 15-21 stand further rejected as being supported by an inadequate written description. Each of these rejections is addressed in turn below.

Nonstatutory Double Patenting

Claims 1-21 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-6 of U.S. Patent No. 6,656,704. As noted by the Examiner, this rejection may be overcome by a timely filed terminal

disclaimer. Applicants will submit such a disclaimer once allowable subject matter has been identified.

35 U.S.C. § 112, first paragraph

Enablement

Claims 1-21 are rejected for lack of enablement. Applicants respectfully traverse this rejection. Because of their differing claim scope, claims 1, 8, and 15, and claims dependent therefrom, are discussed separately.

Claims 8-14

Claims 8-14 are directed to polypeptides consisting of one of six sequences, which encode BIR3 from human or mouse XIAP, HIAP-1, or HIAP-2, wherein the polypeptide is capable of inhibiting apoptosis when expressed in a mammalian cell. In support of the rejection of these claims for lack of enablement, the Examiner discusses four of the Wands factors—claim breadth, guidance provided by the specification, the state of the art, and predictability—and concludes that each of these factors supports the Examiner's position of lack of enablement. Applicants respectfully disagree.

Claim breadth, the first Wands factor discussed by the Examiner, clearly supports applicants position that the claims are enabled; claim 8, the broadest claim in this group, recites only six polypeptides, while each of the other claims recites one polypeptide.

As for the second Wands factor, guidance in the specification, applicants contend that this factor also supports a conclusion that the claims are enabled. First, contrary to the assertions of the Examiner, IAP polypeptides other than full-length polypeptides are taught as being useful for inhibiting apoptosis. The results of experiments using BIR3-containing fragments is presented, for example, at pages 29-31 of the specification, and other BIR-containing fragments are taught to be useful for inhibiting apoptosis (pages 13 and 14). Importantly, fragments containing BIR domains are specifically recited at page 61. The specification also provides guidance for making polypeptides (pages 34-36) and for measuring cell survival or apoptosis (pages 28-31).

In discussing the remaining two Wands factors, state of the art and predictability, the Examiner provides a detailed review of various studies performed by researchers studying IAP polypeptides in support the Examiner's rejection of the claims for lack of enablement. Applicants fail to see how most of the discussion relates to the patentability of the claimed invention. The only statement that relates to BIR3 polypeptides is the one stating "BIR3 domains of XIAP apparently lack caspase-binding capability..." While the Examiner did not provide a citation to a reference, applicants believe the Examiner is

referring to Takahashi et al. (J. Biol. Chem. 273:7787-7790, 1998; hereafter "Takahashi") (Exhibit B), which reported that XIAP BIR2 and not XIAP BIR3 was capable of inhibiting caspase-3 and caspase-7.

Applicants note that contrary to the Examiner's assertion, XIAP BIR3 has been shown to inhibit caspase activity. The Examiner's attention is drawn to Sun et al. (J. Biol. Chem. 275:33777-33781, 2000; hereafter "Sun") (Exhibit A). Sun describes the identification of the minimal region of XIAP that is required for inhibiting caspase-9, a cysteine protease known to play a key role in the execution of apoptosis. As shown in Fig. 1 and stated at page 33779 of Sun, Sun found that "proteins containing only the Bir3 domain were sufficient to inhibit caspase-9..."

In sum, applicants assert that claims 8-14 are enabled, as demonstrated by a careful examination of the Wands factors. The claims are narrowly drawn to six polypeptides, the specification teaches how to make the polypeptides and test them for apoptosis-inhibiting activity, and at least one of the six has been shown to have a caspase-inhibiting activity, which is generally believed to be the mechanism by which IAP polypeptides inhibit apoptosis. Reconsideration and withdrawal of this rejection of claims 8-14 is respectfully requested.

Claims 15-21

Claims 15-21 differ from claims 8-14, discussed above, in that the claimed polypeptides include a BIR3 domain, rather than consist of a BIR3 domain. Nonetheless, the application of the Wands factors to these claims is largely the same as their application to claims 7-14. The main argument set forth by the Examiner is that the inclusion of additional amino acids makes it "highly unpredictable" that any polypeptide would inhibit apoptosis in a mammalian cell. Applicants respectfully disagree.

First, applicants note that the actual findings of researchers undercuts the Examiner's position. As discussed above, in the specification applicants describe experiments demonstrating the ability of full-length IAPs and IAP fragments to inhibit apoptosis. Sun, discussed above and enclosed herewith, further supports applicants' position that the invention is not unpredictable. Even Takahashi supports applicants' position. While referring to BIR2 and not BIR3, Takahashi demonstrates that a fusion protein containing a BIR domain can maintain caspase-inhibiting activity (Takahashi tested XIAP fragments linked to a 239-amino acid glutathione S-transferase protein).

Second, applicants submit that the making and testing of polypeptides containing one of six BIR3 domains is well within the abilities of one skilled in the field of apoptosis. The Examiner states that one would have to engage in "extensive making and testing," but this on its own does not mean that the claims lack enablement. The Federal

Circuit has stated that "a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). In the present case, the necessary experimentation is routine, and the specification provides ample guidance with respect to this experimentation.

For the reasons provided above, applicants respectfully request that the rejection of claims 15-21 for lack of enablement be withdrawn.

Claims 1-7

The final group of claims, claims 1-7, are directed to polypeptides having at least 95% sequence identity to one of the six disclosed BIR3 domains. Applicants direct the Examiner's attention to the applicants' remarks with respect to claims 8-14 and 15-21, which are equally applicable for claims 1-7. Applicants address below the issue of the amount of experimentation, as it appears to be the Examiner's central issue with claims 1-7.

The Examiner contends that the making and testing polypeptides in which 5% of the amino acids are added, substituted, or deleted would not have been considered

routine. The Examiner provides no support for this position, apart from stating that the number of possible proteins is large.

Applicants reiterate that a considerable amount of testing is permissible if it is routine, and submit that the methods for making and testing the claimed polypeptides are all described in the specification and in any event were well within the level of skill of one of ordinary skill in the art as of the priority date of the present application. A practitioner could readily produce and purify any desired polypeptide having 95% identity to one of the six BIR domains without undue experimentation, and could similarly test the polypeptide to determine if it has the necessary apoptosis-inhibiting activity in a mammalian cell. Even if these methods are repeated, they are nonetheless routine.

In view of the foregoing remarks, reconsideration and withdrawal of the rejection of claims 1-21 for lack of enablement is respectfully requested.

Written Description

Claims 1-7 and 15-21 are rejected as being supported by an inadequate written description. According to the Examiner, applicants only describe full-length peptides, and that "[t]he disclosure of single species is rarely, if ever, sufficient to describe a broad genus." Applicants respectfully traverse this rejection.

Applicants first note that that the specification does disclose BIR domain-containing fragments, and not simply full-length peptides as alleged by the Examiner. For example, at page 5, lines 22-25, the specification states "[f]ragments including one or more BIR domains...are also part of the invention." At page 61, lines 17 and 18, the specification states "[p]articularly useful fragments...include, without limitation, the amino acid fragments shown in Table 2 [which lists all of the BIR domains from XIAP, HIAP-1, and HIAP-2]."

Moreover, applicants disagree with the Examiner's statement that a single species rarely if ever is sufficient to describe a broad genus. The M.P.E.P. states that there may be situations where one species adequately supports a genus (M.P.E.P. 2163.05). This is clearly demonstrated in the "Revised Interim Written Description Guidelines Training Materials" posted on the USPTO web site (http://www.uspto.gov/web/menu/written.pdf) (hereafter "the Training Materials"), which describe a claim similar to the claims 1-7 and 15-21 as supported by a single species. After a careful analysis, the Training Materials conclude that this claim satisfies the written description requirement. The claim and the analysis are described in Example 14 of the above document, and applicants summarize them below.

The claim discussed in Example 14 of the Training Materials is as follows:

A protein having SEQ ID NO: 3 and variants thereof that are at least 95% identical to SEQ ID NO: 3 and catalyze the reaction of A-->B.

Like applicants' claims 1-7, this claim is to a protein having at least 95% identity to a reference sequence, the protein being further defined by a biological activity. Like applicants' claims 15-21, this claim is open ended in that the claimed protein may be larger than reference sequence. The Training Materials conclude that the disclosed species is "representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specific catalytic activity." The Training Materials continue to state that "[o]ne of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by members of the genus."

Not only do the Training Materials provide an example when a single species is sufficient to demonstrate possession of an entire genus, the example provided is nearly identical to the present situation. Indeed, claims 1-7 are narrower than is the claim discussed in the Training Materials because these claims are not open ended—the claims are to a polypeptide consisting of (rather than comprising) a sequence having at least 95% sequence identity to one of six reference sequences. Claims 15-21 are also narrower than

the Training Materials claim because these claims, while being open ended, require 100% identity to one of the reference sequences.

For the foregoing reasons, applicants respectfully submit that the rejection of claims 1-7 and 15-21 for lack of an adequate written description be withdrawn.

CONCLUSION

Applicants respectfully submit that this case is in condition for allowance, and such action is respectfully requested. If the Examiner does not concur, a telephonic interview with the undersigned is hereby requested. If there are any additional charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

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EXHIBIT A

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NMR Structure and Mutagenesis of the Third Bir Domain of the Inhibitor of Apoptosis Protein XIAP*

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The inhibitor of apoptosis proteins (IAPs) regulate the caspase family of cysteine proteases, which play an important role in the execution of programmed cell death. Human X-linked inhibitor of apoptosis protein (XIAP) is a potent inhibitor of caspases-3, -7, and -9. Here we show that the Bir3 domain is the minimal region of XIAP that is needed for potent caspase-9 inhibition. The three-dimensional structure of the Bir3 domain of XIAP, determined by NMR spectroscopy, resembles a classical zinc finger and consists of five α-helices, a three-stranded β-sheet, and a zinc atom chelated to three cysteines and one histidine. The structure of the Bir3 domain is similar to that of the Bir2 domain of XIAP but differs from the previously determined structure of the Bir3 domain of MIHB. Based on site-directed mutagenesis, we have identified the regions of the Bir3 domain of XIAP that are important for inhibiting caspase-9. Despite the structural similarities of the Bir2 and Bir3 domain of XIAP, a different set of residues were found to be critical for inhibiting the individual caspases. These results suggest that XIAP inhibits caspase-3 and caspase-9 in a different manner.

Programmed cell death is a tightly regulated process that is critical for normal development and tissue homeostasis and when dysregulated can lead to a variety of diseases such as neurodegenerative disorders and cancer (1). One class of proteins that negatively regulates cell death signaling is the inhibitor of apoptosis proteins (IAPs).[1] IAPs are highly conserved and have been found in many species (2–6). The members of this family are characterized by having one or more baculovirus IAP repeats called Bir domains. Bir domains consist of approximately 70 amino acids that contain the characteristic signature sequence $CX_2CX_{16}HX_6C$ (7, 8). Some IAPs also contain a C-terminal ring finger that contains one zinc atom chelated to three cysteines and one histidine and another zinc ligated to four cysteines (9).

One of the major functions of the IAPs is their ability to bind to and inhibit the cysteine proteases known as caspases (10, 11), which play a key role in the execution of programmed cell death (12). Caspase inhibition by the IAPs can directly explain their antiapoptotic activities. For human XIAP, the region responsible for inhibiting caspases-3 and -7 was localized to a fragment containing the second Bir domain (13). Although the Bir domain was necessary for caspase-3 inhibition, residues outside of the Bir2 domain were also found to be critical for inhibiting caspase-3 (14). On the basis of site-directed mutagenesis and NMR studies on the interaction of XIAP with caspase-3, it was postulated that the residues N-terminal to the Bir2 domain of XIAP bind to the active site of this enzyme (14).

XIAP also inhibits caspase-9. However, a different portion of XIAP is involved. Recently, Deveraux *et al.* (15) have shown that it is the Bir3 and ring finger of XIAP that potently inhibits caspase-9. Since neither the ring finger nor the Bir3 domain of XIAP was sufficient to inhibit caspase-9, it was postulated that the ring finger may function to stabilize the Bir3 domain in a conformation required for caspase-9 inhibition (15).

Three-dimensional structures of the Bir2 domain of XIAP (14) and the Bir3 domain of MIHB (cIAP-1) (16) have been reported. The structure of the Bir2 domain of XIAP resembles a classical zinc finger and consists of four α-helices and a three-stranded antiparallel β-sheet. The structure of the Bir3 domain of MIHB, on the other hand, was reported (16) to be an α-helical protein with a fold not previously observed for zinc-binding proteins.

In this paper, we have identified the minimal region of XIAP that is required for inhibiting caspase-9. In contrast to earlier studies (15), we find that the ring finger is unnecessary for potent caspase-9 inhibition. The Bir3 domain alone is sufficient. We have also determined the three-dimensional structure of the Bir3 domain of XIAP. The structure of this protein is compared with the previously determined structures of the Bir2 domain of XIAP and Bir3 domain of MIHB. Finally, on the basis of site-directed mutagenesis, we have identified amino acids of XIAP that are important for caspase-9 inhibition.

MATERIALS AND METHODS

Protein Preparation—XIAP (residues 241–497) was cloned from a Jurkat cDNA library into the pET-28b vector (Novagen). C-terminal deletions were created by introducing a stop codon at the appropriate positions. Site-directed mutants were prepared using the Quick-Change mutagenesis kit (Stratagene). The coding region in all of the plasmids was confirmed by sequencing.

The protein (residues 241–356) used in the structure determination was expressed in the *Escherichia coli* strain BL21(DE3) (Novagen). Uniformly $^{15}N/^{13}C$-and ^{15}N-labeled proteins were prepared by growing bacterial cells in a minimal medium containing a trace amount of

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The atomic coordinates and structure factors (code 1F9X) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[1] The abbreviations are: IAP, inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis protein; Bir, baculovirus IAP repeat; NOE, nuclear Overhauser effect; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; HSQC, heteronuclear single quantum correlation.

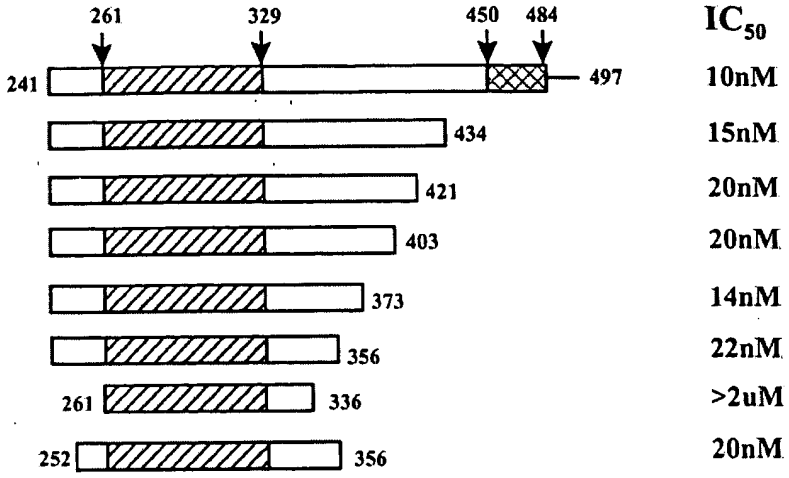

FIG. 1. **Schematic representation of the truncated XIAP proteins along with their inhibition constants (IC_{50} values) against caspase-9.** The Bir3 and ring finger domains are indicated with *hatched* and *cross-hatched boxes*, respectively. The amino acids are *numbered* according to the full length protein. All proteins contain an N-terminal His tag.

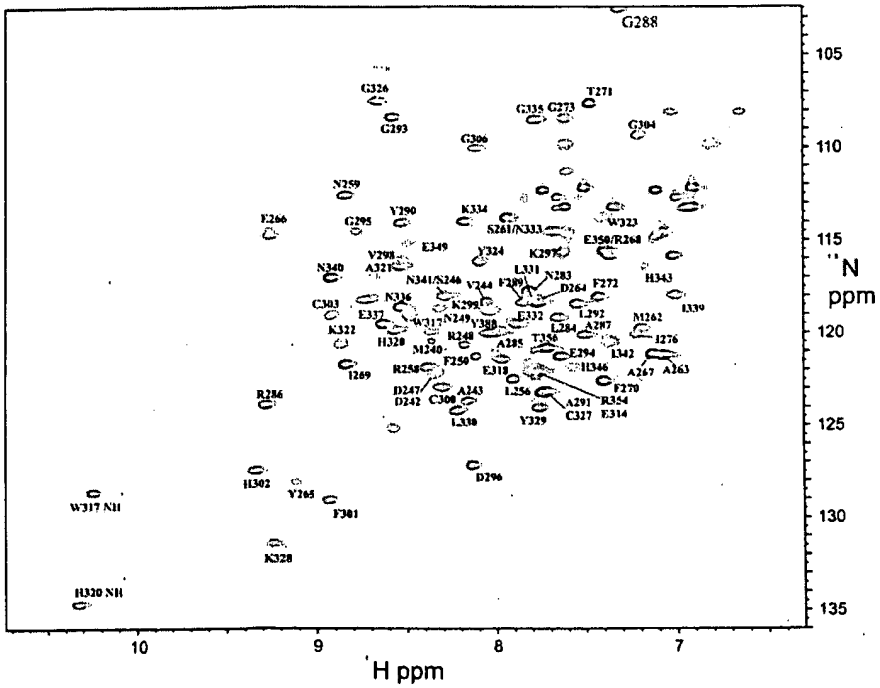

FIG. 2. **^{15}N-^{1}H HSQC spectrum of the Bir3 domain (residues 241–356) of human XIAP.** Assignments for the backbone NH peaks and side chains of Trp-317 and His-320 peaks are labeled.

$Zn(Ac)_2$ and $^{15}NH_4Cl$ with or without uniformly ^{13}C-labeled glucose. Recombinant proteins were purified using a nickel-nitrilotriacetic acid column (Qiagen) followed by gel filtration (Amersham Pharmacia Biotech). The N-terminal His tag was cleaved with thrombin (Novagen), leaving four extra residues (GSHM) at the N terminus.

NMR Spectroscopy—NMR samples contained 1.7 mM protein in 50 mM bis-Tris-d_{19} (pH = 7.2), 300 mM KCl, 50 μM $Zn(Ac)_2$, and 1 mM dithiothreitol. NMR spectra were acquired at 30 °C on a Bruker 500-, 600-, or 800-MHz NMR spectrometer. The ^{1}H, ^{15}N, and ^{13}C resonances of the backbone and side chains were assigned using a standard set of double and triple resonance experiments (17). The prochiral methyl groups of Val and Leu were stereospecifically assigned by recording an HSQC spectrum of a 15% ^{13}C-labeled sample of the protein (18). Distance restraints were derived from ^{15}N- and ^{13}C-resolved three-dimensional NOE spectroscopy. Slowly exchanging amide protons were identified from a series of ^{1}H-^{15}N HSQC spectra after the H_2O buffer was exchanged to a buffer containing $^{2}H_2O$. Residual dipolar couplings were measured as described previously (19, 20) using a $^{13}C/^{15}N$-labeled sample of the protein dissolved in a solution containing phage at a concentration of 17 mg/ml.

Structure Calculations—In the first step of the structure calculations, 577 unambiguous NOEs, 62 hydrogen bond restraints, and 84 ϕ or φ angular restraints derived from the TALOS program (21) were incorporated into a torsion angle dynamics (22) and simulated annealing protocol (23) using the program CNX (MSI Inc., San Diego, CA). A single family of low energy structures was obtained from these calculations that defined the overall fold. Refinement of these initial structures was accomplished using the ARIA protocol (24). New NOE assignments were accepted from ARIA if they agreed with the manually derived NOEs. From 8 iterations, 1280 unambiguous and 904 ambiguous NOE assignments were derived. In the final round of refinement, 46 N-H, 26 C^{α}-H^{α}, 30 H^{N}-C', and 43 C^{α}-C' residual dipolar couplings were used along with the NOE-derived distance restraints, angular restraints from TALOS, and hydrogen bond restraints.

Caspase Inhibition Assays—Caspase-9 inhibition was measured using a truncated form of the enzyme that lacks the N-terminal caspase recruitment domain. This form of the enzyme has the full catalytic activity and the same substrate specificity as the full-length protein.[2] LEHD-7-amido-4-methylcoumarin (Calbiochem) was used as the substrate in the caspase-9 assay. The reaction mixture (200 μl) contained 0.55 nM caspase-9 and 25 μM substrate in the assay buffer (20 mM Hepes, 0.1 mM EDTA, 0.1% Chaps, 10% sucrose, and 5 mM dithiothreitol) at pH 6.5. The activity was measured at 30 °C by monitoring the formation of fluorescent 7-amido-4-methylcoumarin over time at 460 nm using an excitation wavelength of 360 nm. IC_{50} values were calculated from the percentage of inhibition measured using various concentrations of XIAP proteins. XIAP proteins were prepared as 8 μM stock

[2] J. S. Krebs and J. C. Wu, unpublished data.

solutions in the assay buffer and subsequently serially diluted to give a 10-point curve. In each assay, a control inhibitor (DEVD-aldehyde) was included.

RESULTS AND DISCUSSION

Minimal Region of XIAP That Inhibits Caspase-9—To identify the minimal region of XIAP that inhibits caspase-9, a series of truncated XIAP proteins were expressed in bacteria, purified, and tested for their ability to inhibit recombinant caspase-9 *in vitro* (Fig. 1). We also tested the ability of these proteins to inhibit a panel of caspases (caspase-1, -3, -6, -7, -8, and -9). Consistent with earlier results (15), a fragment of XIAP containing the Bir3 domain and ring finger was found to specifically inhibit caspase-9. However, in contrast to this earlier study, we found that the ring finger is unnecessary for caspase-9 inhibition. Indeed, much smaller proteins containing only the Bir3 domain were sufficient to inhibit caspase-9 (Fig. 1). The reason for this apparent discrepancy was investigated by characterizing the shorter protein (residues 261–336) used in the previous work by NMR spectroscopy. The amide protons observed in an ^{15}N-^{1}H correlation spectrum of the uniformly ^{15}N-labeled protein appeared in a relatively narrow frequency range. These results strongly suggest that the protein is unfolded and can explain why the short protein was unable to inhibit caspase-9. The smallest protein that was folded and potently inhibited caspase-9 contained residues 241–356 (Fig. 1). This protein was chosen for the structural studies.

Structure Determination of XIAP (Residues 241–356)—The $^{1}H/^{15}N$ HSQC spectrum of the Bir3 domain (residues 241–356) was well dispersed, which allowed the signals to be readily assigned (Fig. 2). A total of 2475 NMR-derived restraints were used in the structure calculations (Table I). Fig. 3 depicts the ensemble of 20 structures of the Bir3 domain of XIAP that were

TABLE I

Structural statistics and root mean square deviations for the 20 NMR-derived structures of the Bir3 domain of XIAP

⟨SA⟩ is the ensemble of 20 NMR-derived structures for XIAP (residues 241–356); $\langle SA \rangle_r$ is the energy-minimized mean structure.

	CNX potential energies	
	⟨SA⟩	$\langle SA \rangle_r$
	kcal mol^{-1a}	
E_{tot}	288.0 ± 19.4	286.8
E_{bond}	9.1 ± 1.2	9.3
E_{ang}	120.2 ± 7.3	127.6
E_{impr}	20.0 ± 2.5	18.4
E_{vdw}	27.9 ± 4.2	33.4
E_{cdih}	2.7 ± 0.7	2.7
E_{noc}	30.8 ± 8.1	20.0
E_{sani}	76.4 ± 5.0	73.2
Cartesian coordinate root mean square deviation (Å)[b]	N, Cα, C′	All heavy
Residues 255–345	0.70 ± 0.07	1.41 ± 0.16

[a] Energies were calculated by using CNX. The F_{repel} function was used to simulate van der Waals interactions with a force constant of 4 kcal mol^{-1} Å^{-4} with the atomic raddii set to 0.8 times their CHARMM values (27). Torsional restraints were derived from TALOS with 2× standard deviation and a minimum error of ±15 °. No torsional angle restraints were violated by more than 5 ° in any of the final structures. No NOE restraint was violated by greater than 0.4 Å in any structure. Residual dipolar coupling restraints were employed using the SANI potential energy function with a force constant of 0.5.

[b] Atomic root mean square deviation between the 20 NMR structure ensemble structures and the mean structure after superposition of all backbone atoms (N, Cα, C′) or all non-hydrogen atoms.

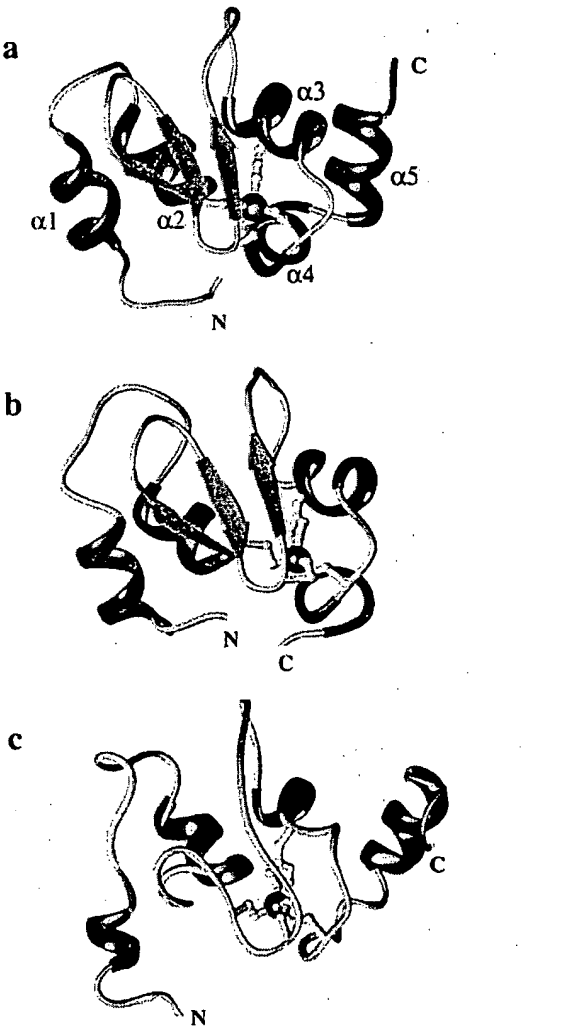

FIG. 4. **Ribbon (25) depiction of the structural comparison of the Bir3 domain of XIAP (residues 255–346) (*a*), the Bir2 domain of XIAP (residues 155–254) (*b*), and the Bir3 domain of MIHB (residues 268–356) (*c*).** The α-helices are shown in *red*, β-sheets in *green*, zinc in *magenta*, and the side chains of the residues that chelate zinc are colored *yellow*.

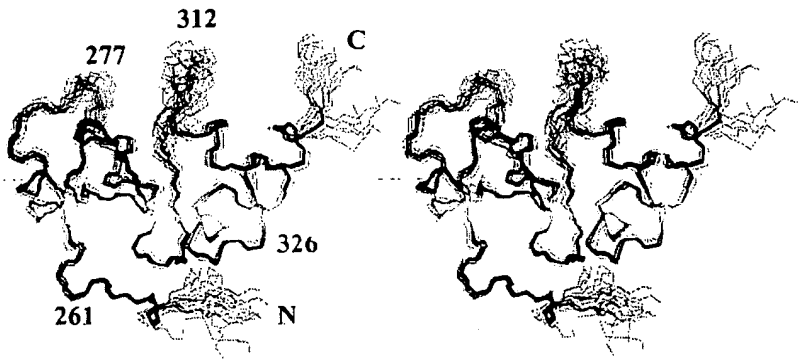

FIG. 3. **Stereoview of the backbone of 20 NMR-derived structures of the Bir3 domain (residues 252–346) of XIAP.**

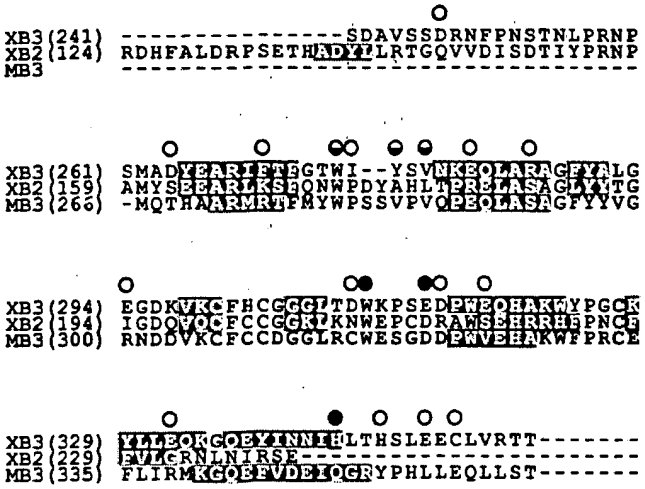

FIG. 5. **Sequence alignment of the Bir3 domain of XIAP (*XB3*), the Bir2 domain of XIAP (*XB2*), and the Bir3 domain of MIHB (*MB3*).** The secondary structural elements for each protein are color-coded in *red* for the α-helices and *green* for the β-strands. The *circles* above the sequence denote the residues that have been mutated in the Bir3 domain of XIAP and tested for caspase-9 inhibition. *Open circles* indicate no significant change in caspase-9 inhibition, the *half-filled circles* indicate more than a 3-fold decrease in caspase-9 inhibition, and the *filled circles* indicate the complete loss ($IC_{50} \geq 1$ μM) of caspase-9 inhibition.

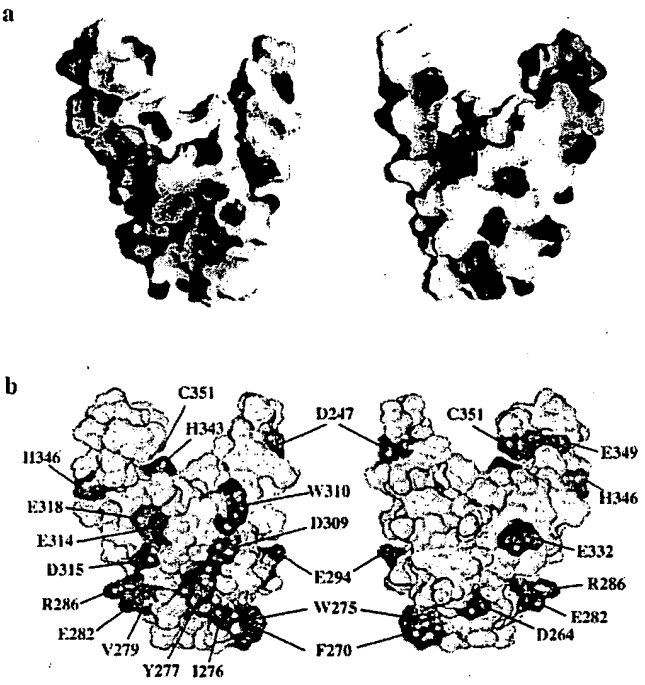

FIG. 6. **(*a*) Two views of the electrostatic potential surface of the averaged minimized NMR structure of XIAP (245–356) prepared using the GRASP program (26).** The *red* areas correspond to the negatively charged regions, and the *blue* areas represent the positively charged portions of the protein. (*b*) Surface representation of the Bir3 domain of XIAP in the same orientation as shown in Fig. 4*a*. Residues with little effect on caspase-9 inhibition are colored *green*, whereas those residues that displayed a marked loss in their ability to inhibit caspase-9 ($IC_{50} > 1$ μM) are colored *magenta*.

derived from the NMR data. The root mean square deviation about the mean coordinate positions in the ensemble for residues 255–345 is 0.70 ± 0.07 Å for the backbone atoms and 1.41 ± 0.16Å for all heavy atoms. The N terminus (residues 241–254) and C terminus (residues 345–356) corresponding to the amino acids outside of the Bir3 domain are disordered. However, the Bir3 domain is well defined except for residues Ile-276 through Asn-280 and Thr-308 through Glu-314. The amide protons of the ill-defined residues were not observed in any of the NMR spectra, suggesting that these signals are exchange broadened.

The structure of XIAP (241–356) consists of five α-helices, three β-strands, and a zinc atom, which is coordinated to the conserved residues Cys-300, Cys-303, Cys-327, and Nε2 of H320 (Figs. 4*a* and 5). The first α-helix (residues 265–272) runs antiparallel to the second (residues 282–286). These two α-helices are followed by the conserved residues Ala-287 and Gly-288, which form a sharp turn, leading to the three-stranded antiparallel β-sheet. The β-sheet is followed by α3 (residues 316–322), α4 (residues 327–333), and α5 (residues 336–342), which are nearly perpendicular to one other. α5 packs against α3 and forms a hydrophobic cluster involving Trp-317, Ile-339, and Ile-342. These interactions appear to be important and can explain why the shorter protein (residues 261–336) lacking the C-terminal α-helix is unfolded and unable to inhibit caspase-9.

The surface of the Bir3 domain is depicted in Fig. 6. In addition to the conserved hydrophobic residues (Phe-272, Leu-284, Phe-289, Val-298, Leu-330) that form the core of the Bir3 domain, there are several hydrophobic residues that are exposed on the surface, including Phe-270, Trp-275, Val-279, and Trp-310. Also noteworthy is a patch of negatively charged residues on the surface, which is composed of Glu-314, Asp-315, and Glu-318 (Fig. 6). On the other side of the protein, there is a cluster of positively charged residues (Arg-268, Arg-258, and Lys-299).

Structural Comparison to Other Bir Domains—The structure of the Bir3 domain of XIAP is very similar to that of the Bir2 domain of XIAP (Figs. 4 and 5). They both contain a three-stranded antiparallel β-sheet, α-helices of similar lengths and orientations, a zinc atom chelated by three conserved cysteines and a histidine, and a hydrophobic core made up of highly conserved residues. The root mean square deviation between the Bir3 and Bir2 domain of XIAP is 1.5 Å for 49 residues located within the conserved secondary structural elements. The only difference is the presence of an extra C-terminal α-helix in the Bir3 domain. The residues that form the C-terminal α-helix are highly conserved in Bir3 domains but not in other Birs, suggesting that the C-terminal α-helix may be uniquely found in Bir3 domains. Indeed, the Bir3 domain of MIHB (Fig. 4*c*) contains a C-terminal α-helix corresponding to the one found in the Bir3 domain of XIAP. In addition, like the Bir2 and Bir3 domain of XIAP, a zinc is chelated to three cysteines and a histidine. However, unlike the Bir domains of XIAP, the Bir3 domain of MIHB lacks a β-sheet, is missing α4, and has shorter α-helices for α1 and α3 (Figs. 4 and 5). These structural differences are surprising in view of the high sequence homology observed for the Bir domains (8).

TABLE II
Inhibition constants (IC_{50}) of XIAP mutants against caspase-9

Name	IC_{50}	Name	IC_{50}
	nM		*nM*
Bir3-RF (241–497)	11 ± 4	E294A	36 ± 10
Bir3 (241–356)	22 ± 9	D309A	26 ± 5
D247A	16 ± 2	W310A	>2 μM
D264A	31 ± 7	E314S	>2 μM
F270K	46 ± 12	D315A	64 ± 18
W275A	145 ± 3	E318A	31 ± 14
I276A	38 ± 11	E332A	38 ± 9
Y277A	84 ± 3	E349A	31 ± 6
V279A	161 ± 21	H343A	>1μM
E282A	37 ± 11	H346A	24 ± 6
R286A	34 ± 10	C351A	19 ± 3

Amino Acids Important for Caspase-9 Inhibition—In an ear-

lier study (14), we found that the residues of XIAP important for caspase-3 inhibition were located in a linker region between the Bir1 and Bir2 domains that contained a sequence (DISD) resembling the residues found in substrates and inhibitors of caspase-3. The substrate specificity for caspase-9 is different and consists of the consensus sequence (I/L/V)-*X*-*X*-D. Interestingly, a stretch of amino acids (VSSDRNI) that resembles the substrate consensus sequence for caspase-9 was found N-terminal to the Bir3 domain of XIAP. To determine whether these residues were important for caspase-9 inhibition, a truncated protein that lacked these residues was prepared. The truncated protein (residues 252–356) potently inhibited caspase-9 (Fig. 1). Furthermore, the mutation of Asp-247 to alanine, which would be predicted to alter caspase-9 inhibition if this region were important, shows no effect (IC_{50} ~17 nM, Table II). Therefore, unlike the importance of the residues N-terminal to the Bir2 domain for caspase-3 inhibition, the analogous region N-terminal to the Bir3 domain is not required for the inhibition of caspase-9.

Several other mutant proteins were prepared and tested for their ability to inhibit caspase-9 (Fig. 6, Table II). Most of the mutant proteins showed no effect and potently inhibited caspase-9. However, mutation of Glu-314 to a serine completely abolished caspase-9 inhibition. In addition, an attenuation of casapse-9 inhibition was observed for W275A and V279A, and a complete loss of caspase-9 inhibition was observed for the W310A and H343A mutant proteins (Table II).

In order to check whether the mutant proteins were properly folded, ^{15}N-labeled proteins were prepared for those mutants that displayed a large loss of caspase-9 inhibition, and their ^{1}H,^{15}N-HSQC spectra were compared with a spectrum of the wild-type protein. With the exception of the W275A mutant protein, the spectra of the mutant proteins only displayed minor chemical shift differences compared with the wild-type protein, suggesting that the structures are very similar.

As shown in Fig. 6, the residues that have the largest effect on caspase-9 inhibition (Glu-314, Trp-310, His-343) are all located in the same region of the protein. Glu-314 is part of a cluster of acidic residues that make up a negatively charged patch on the surface of the protein (Fig. 6). However, the other acidic residues of this patch (Asp-315 and Glu-318) do not appear to be important for caspase-9 inhibition because, when mutated to alanine, they show no loss of inhibitory potency (Table II). Trp-310 and His-343 are located on the edge of a basic patch that forms a shallow pocket on the protein surface. This site appears to be the region of XIAP that interacts with caspase-9.

Conclusions—The IAPs play a critical role in the regulation of programmed cell death. One of their major functions is to bind to and inhibit the caspases, which if left unchecked could lead to massive cell death. XIAP potently inhibits both caspase-3 and caspase-9. The inhibition of caspase-3 is accomplished by binding of the Bir2 domain and residues N-terminal to the Bir2 domain to the enzyme (14). In contrast, as shown here, only the Bir3 domain of XIAP is required to inhibit caspase-9. Residues N-terminal to the Bir3 domain or the ring finger are unnecessary for potent caspase-9 inhibition. The structure of the Bir3 domain of XIAP was found to be similar to the previously determined structure of the Bir2 domain of XIAP (14). Despite these structural similarities, however, the amino acid residues in the Bir3 domain found to be important for caspase-9 inhibition are different from those necessary to inhibit caspase-3. Thus, XIAP employs a distinctly different mechanism to inhibit different caspases.

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EXHIBIT B

Communication

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A Single BIR Domain of XIAP Sufficient for Inhibiting Caspases*

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The inhibitor of apoptosis proteins (IAPs) constitute an evolutionarily conserved family of homologous proteins that suppress apoptosis induced by multiple stimuli. Some IAP family proteins, including XIAP, cIAP-1, and cIAP-2, can bind and directly inhibit selected caspases, a group of intracellular cell death proteases. These caspase-inhibiting IAP family proteins all contain three tandem BIR domains followed by a RING zinc finger domain. To determine the structural basis for caspase inhibition by XIAP, we analyzed the effects of various fragments of this IAP family protein on caspase activity *in vitro* and on apoptosis suppression in intact cells. The RING domain of XIAP failed to inhibit the activity of recombinant caspases-3 or -7, whereas a fragment of XIAP encompassing the three tandem BIR domains potently inhibited these caspases *in vitro* and blocked Fas (CD95)-induced apoptosis when expressed in cells. Further dissection of the XIAP protein demonstrated that only the second of the three BIR domains (BIR2) was capable of binding and inhibiting these caspases. The apparent inhibition constants (K_i) for BIR2-mediated inhibition of caspases-3 and -7 were 2–5 nM, indicating that this single BIR domain possesses potent anti-caspase activity. Expression of the BIR2 domain in cells also partially suppressed Fas-induced apoptosis and blocked cytochrome *c*-induced processing of caspase-9 in cytosolic extracts, whereas BIR1 and BIR3 did not. These findings identify BIR2 as the minimal caspase-inhibitory domain of XIAP and indicate that a single BIR domain can be sufficient for binding and inhibiting caspases.

IAP[1] family proteins have been identified in baculoviruses, *Drosophila*, and humans, where they function as suppressors of apoptosis (1–8). In all cases examined thus far, apoptosis has been shown to depend upon the activation of a group of intracellular cysteine proteases with specificity for aspartic acid in the P_1 position of substrates, called caspases (reviewed in Ref. 20). The human XIAP, cIAP-1, and cIAP-2 proteins can bind to and directly inhibit selected caspases. The caspases identified as targets of these IAP family proteins thus far include caspases-3, -7, and -9 but not caspases-1, 2, 8, or 10 (9, 10, 24).[2]

XIAP, cIAP-1, and cIAP-2 all contain three tandem copies of a ~70-amino acid motif termed the BIR (baculovirus IAP repeat) domain, reflecting the historical discovery of IAP family proteins originally in baculoviruses by Miller and colleagues (1, 11). These IAP family proteins also contain a RING zinc finger domain near their C termini. The IAP family protein NAIP, in contrast, contains three tandem BIRs but lacks the RING domain (3). An additional member of the human IAP family, survivin, contains only a single BIR domain but nevertheless can suppress apoptosis in cells (8). The IAP family proteins of *Drosophila* and the insect-infecting baculoviruses all contain two or three tandem copies of the BIR domain followed by a RING domain (1, 2, 11). Deletional analyses of the *Drosophila* and baculovirus IAP family proteins have produced conflicting results, with some studies suggesting a critical role for both the BIR and RING domains and others implying that BIRs are directly involved in apoptosis suppression with the RING motif functioning possibly as a negative regulatory domain (2, 12).

The structural similarities and differences among IAP family proteins prompted us to address the question of what constitutes the minimal domain necessary for inhibition of caspases and suppression of apoptosis. Using the human XIAP protein as a model, we determined that only one of the three BIR domains present within this protein was clearly capable of inhibiting caspases-3 and -7, as well as suppressing apoptosis when expressed in cells. These observations demonstrated that a single BIR domain can be sufficient for binding and inhibiting caspases and imply that all BIR domains may not be functionally equivalent, despite their extensive amino acid sequence identity.

MATERIALS AND METHODS

Plasmid Constructions—Plasmids encoding fragments of the XIAP protein, including BIR1+2+3 (residues 1–336), RING (residues 337–497), BIR1 (residues 1–123), BIR2 (residues 124–260), BIR3 (residues 261–336), BIR2+3 (residues 124–336), and BIR1+2 (residues 1–260) were created by a one-step polymerase chain reaction method employing a plasmid encoding wild-type XIAP as the template (16) and using 5′-GGAATTCATGACTTTTAACAGTTTTGAAG-3′ (BIR1+2+3, BIR1 and BIR1+2), 5′-GGAATTCCAGCTGCAAGAGGAGAAGCTTTG-3′ (RING), 5′-GGAATTCAGAGATCATTTTGCCTTAGACA-3′ (BIR2 and BIR2+3), or 5′-GGATTCTCCATGGCAGATTATGAAGC-3′ (BIR3) as the forward primer and 5′-CTCTCGAGCATGCCTACTATAGAGTTAGA-3′ (RING), 5′-CTCTCGAGCTACTTGTCCCTTCTGTTCTAACAG-3′ (BIR1+2+3, BIR3 and BIR2+3), 5′-CTCTCGAGCTATGGATTTCTTGGAAGATTTGTTG-3′ (BIR2 and BIR1+2(, or 5′-CTCTCGAGTTACCCCATGGATCCCAGATAGTTTTCAACT-3′ (BIR1) as the reverse primer. The BIR1+3 mutant protein (see Fig. 1) was prepared by ligating the BIR1 and BIR3 polymerase chain reaction fragments di-

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[1] The abbreviations used are: IAP, inhibitor of apoptosis protein; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; AFC, 7-amino-4-trifluoromethyl-coumarin; DEVD, Asp-Glu-Val-Asp; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; GFP, green fluorescence protein; DAPI, 4′,6-diamidino-2-phenylindole.

[2] R. Takahashi, Q. Deveraux, I. Tamm, K. Welsh, N. Assa-Munt, G. S. Salvesen, and J. C. Reed, submitted for publication.

gested with *Eco*RI+*Nco*I and *Nco*I+*Xho*I, respectively. All the fragments were subcloned into pGEX4T-1 (Pharmacia Biotech Inc.) and/or pcDNA3-myc (10) by digestion with *Eco*RI and *Xho*I. The proper construction of all the plasmids was confirmed by DNA sequencing.

Production of GST Fusion Proteins—For production of BIR1+2+3 and RING, pGEX4T-1-XIAP mutant plasmids were introduced into *Escherichia coli* strain BL21(DE3) containing the plasmid pT-Trx (13). BIR1, BIR2, and BIR3 were produced in XL-1 blue cells (Stratagene, Inc). The GST fusion proteins were prepared from the soluble fraction upon induction with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 12 h, affinity purified using glutathione-Sepharose, and then dialyzed against phosphate-buffered saline. Coomassie staining analysis of GST fusion proteins following SDS-PAGE revealed ≥80% intact protein in all cases except BIR3 (see text). His_6-tagged caspase-3 and -7 recombinant proteins are prepared as described (14, 15).

Enzyme Assays—Caspase-3 and -7 activities were assayed by release of 7-amino-4-trifluoromethyl-coumarin (AFC) from Asp-Glu-Val-Asp (DEVD)-AFC using continuous reading instruments as described (15). Apparent K_i values were determined using 0.1–0.3 nM caspases and a range of concentrations of recombinant XIAP or XIAP deletion mutants, as described previously (9, 10).

In Vitro Caspase Binding Assays—2 μg of His_6-tagged caspases-3 and -7 were immobilized on 4 μl of Ni^{2+}-Sepharose and incubated with 5 μl of *in vitro* translated, [^{35}S]L-methionine-labeled BIR1, BIR2, BIR2+3, BIR1+2, or BIR1+3 proteins, that were prepared from pcDNA3-myc (XIAP mutants) templates using a coupled transcription/translation system involving rabbit reticulocyte lysates (TNT-lysate, Promega, Inc.) and T7 RNA polymerase. Incubations were performed in 0.1 ml of caspase buffer, consisting of 50 mM Tris-Cl (pH 7.5), 100 mM KCl, 10% sucrose, 0.1% CHAPS, and 5 mM dithiothreitol at 4 °C for 1 h. The beads were washed with 1 ml of caspase buffer three times before resuspending samples in boiling Laemmli buffer and analyzing by SDS-PAGE using 750 mM Tris/15% polyacrylamide gels and autoradiography.

Cytochrome c Activation of Caspases in Cytosolic Extracts—Cytosolic extracts were prepared using 293 cells as described (10). ^{35}S-Labeled *in vitro* translated pro-caspase-9 (1 μl) was added to 5 μl of extracts, and caspase activation was initiated by addition of 10 μM horse heart cytochrome *c* (Sigma) and 1 mM dATP at 30 °C.

Cell Transfections and Apoptosis Assays—Subconfluent 293 cells were transfected in 3-cm-diameter wells using a calcium phosphate method with 0.5 μg of the green fluorescence protein (GFP) marker plasmid pEGFP (CLONTECH), 1 μg of pCMV-Fas expression plasmid (16), and 5 μg of either pcDNA3-myc-XIAP mutant plasmids or the control plasmid pcDNA3-myc tag. After culturing ~1 day, both floating and attached cells were collected, fixed in 3.7% formaldehyde in phosphate-buffered saline for 10 min, and stained with 10 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI). The percentage of apoptotic cells revealed by DAPI staining was counted among the GFP-positive cells by fluorescence microscopy.

Immunoblot Assays—Transfected 293 cells were lysed in 10 mM HEPES (pH 7.5), 142 mM KCl, 1 mM EGTA, 1 mM dithiothreitol, 0.2% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride and used for immunoblot analysis (9, 10). Samples were normalized for total protein content (25 μg), and immunoblot analysis was performed using 750 mM Tris/12–15% polyacrylamide gels and anti-Myc tag monoclonal antibody 9E10 (Santa Cruz, Inc.) with emission chemiluminescence-based detection (9, 10).

RESULTS AND DISCUSSION

Fig. 1 depicts the fragments of XIAP that were expressed as GST fusion proteins in bacteria and affinity purified. The roles of the BIR and RING domains in inhibition of caspases were examined using a C-terminal truncation mutant of XIAP that contained the three tandem BIRs but lacked the downstream linker region and RING domain (BIR1+2+3) and a XIAP fragment lacking the N-terminal BIR domain but retaining the linker and RING regions (RING). Comparison were made of the effects of the BIR1+2+3 (residues 1–336) and RING (residues 337–497) fragments on recombinant caspase-3- or caspase-7-mediated cleavage of a fluorigenic tetrapeptide substrate, DEVD-AFC, *in vitro*. As shown in Fig. 2 (*A* and *B*), the BIR1+2+3 fragment of XIAP inhibited DEVD-AFC substrate cleavage by caspases-3 and -7, whereas the C-terminal fragment of XIAP containing the RING domain did not.

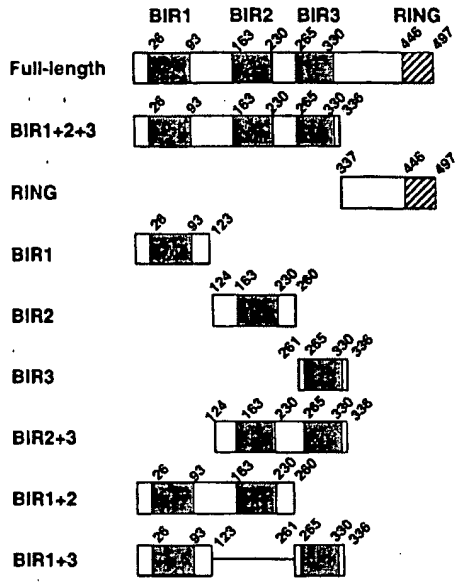

FIG. 1. **Structure of XIAP mutants.** The various XIAP fragments that were expressed with either N-terminal GST or Myc-epitope tags are depicted.

Next, fragments of XIAP containing each of the individual BIR domains (Fig. 1) were expressed in bacteria, purified, and tested for inhibition of recombinant caspases-3 and -7 *in vitro*. Of the three recombinant BIR domains, only BIR2 prevented DEVD-AFC substrate hydrolysis by caspases-3 and -7 (Fig. 2, *C* and *D*). However, because less than half of the purified BIR3 domain appeared to be intact, an additional BIR3-containing fragment of XIAP, which proved to be more stable and which combined BIR1 and BIR3 (Fig. 1), was also produced and tested. This BIR1+3 fragment of XIAP was ineffective at inhibiting caspases-3 or -7 *in vitro*, whereas BIR1+2 and BIR2+3 fragments that contained BIR2 completely suppressed hydrolysis of DEVD-AFC under the conditions of these *in vitro* protease assays (not shown).

Determination of the apparent K_i for inhibition of recombinant caspases-3 and -7 by the BIR2 fragment of XIAP (residues 124–260) produced values of 1–5 nM, thus demonstrating potent suppression of these proteases (Table I). Similarly, the BIR1+2 and BIR2+3 fragments of XIAP that contained the BIR2 domain also inhibited caspases-3 and -7 with K_i values of 1–3 nM. Thus, although not as potent as the full-length XIAP molecule that has been reported to inhibit these proteases with K_i values of 0.2–0.7 nM (10), the BIR2 domain nevertheless retains substantial anti-caspase activity. By comparison, the commonly used tetrapeptide inhibitor DEVD-aldehyde has been reported to inhibit caspases-3 and -7 with K_i values of 0.5–3.5 nM (17–19).

In addition to exploring their ability to inhibit the activity of caspases, the binding of recombinant caspases-3 and -7 to BIR2 and other fragments of XIAP was examined using His_6-tagged caspases immobilized on Ni^{2+} resin and ^{35}S-labeled *in vitro* translated XIAP fragments. All BIR2-containing fragments of XIAP, including BIR2, BIR1+2, and BIR2+3, bound to immobilized caspases-3 and -7 (Fig. 3*A*). In contrast, fragments of XIAP lacking BIR2, including BIR1 and BIR1+3, failed to bind these caspases. Note that under these *in vitro* conditions where high concentrations of proteases are involved, caspase-3 cleaved the BIR2-containing fragments of XIAP, causing slightly faster migration of the ^{35}S-labeled XIAP protein fragments in gels, whereas caspase-7 did not cleave these same XIAP fragments (Fig. 3*A*). Thus, cleavage does not necessarily correlate with enzyme inhibition. Moreover, no evidence has

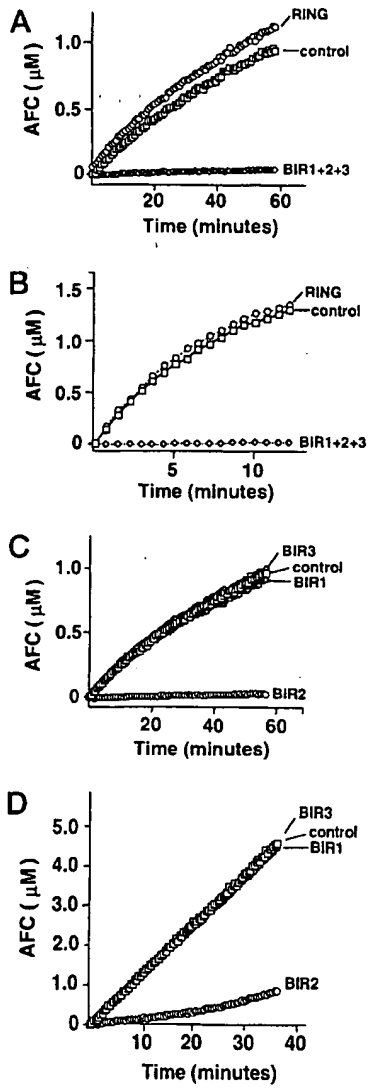

FIG. 2. ***In vitro* inhibition of recombinant caspases-3 and -7 by BIR2 domain of XIAP.** In *A* and *B*, the GST fusion proteins representing the BIR1+2+3 (residues 1–336) and RING (residues 337–497) domains of XIAP were added at 0.1 μM to either 0.1 nM caspase-3 (*A*) or 0.3 nM caspase-7 with 100 μM DEVD-AFC substrate (*B*). Release of AFC fluorigenic label was monitored continuously over time. In *C* and *D*, GST fusion proteins containing the BIR1 (residues 1–123), BIR2 (124–260), or BIR3 (261–336) domains were added at 0.1 μM to either caspase-3 (*C*) or caspase-7 (*D*), and DEVD-AFC hydrolysis was measured as above.

TABLE I

Apparent inhibition constants ($K_{i\ app}$ (nM)) for GST-XIAP fragments measured against caspases-3 and -7

Each purified GST fusion protein was incubated at concentrations ranging from 0.7 to 20 nM with either caspase-3 (50 pM) or caspase-7 (300 pM). Progression curves of caspase activity were then measured at 37 °C for 30 min using 100 μM DEVD-AFC as a substrate. $K_{i\ app}$ was calculated as previously described (10). Neither BIR1 nor BIR3 showed significant influence on caspase-3 or caspase-7 activity even at 10 μM; thus inhibition constants for these polypeptides were estimated to be greater than 1 μM.

	K_i				
	BIR2 (124–260)	BIR1+2 (1–260)	BIR2+3 (124–336)	BIR1 (1–123)	BIR3 (261–336)
					nM
Caspase-3	5	3	3	>1000	>1000
Caspase-7	2	1	1	>1000	>1000

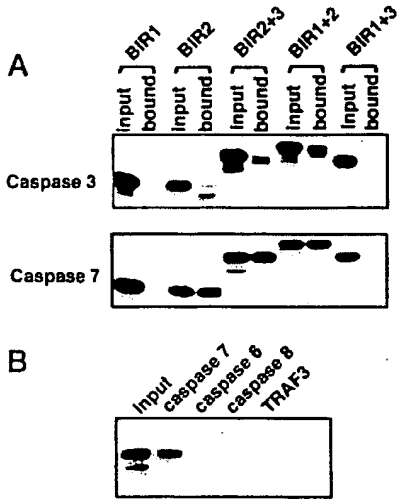

FIG. 3. ***In vitro* binding of BIR2 domain of caspases-3 and -7.** His_6-tagged caspases-3 or -7 (2 μg) were immobilized on Ni^{2+} resin and incubated with *in vitro* translated ^{35}S-labeled Myc-tagged XIAP fragments as indicated. After extensive washing, the bound proteins were eluted into boiling Laemmli buffer and analyzed by SDS-PAGE. As a control, an equivalent amount of input ^{35}S-XIAP protein fragments was loaded directly into gels. In *A*, cleavage of BIR2-containing fragments by caspase-3 reduced the amounts of XIAP-protein fragments recovered and accounts for the slightly faster mobility of these fragments in gels, compared with input (control) XIAP fragments. In *B*, the BIR2 fragment was tested for binding to His_6-caspases-6, -7, and -8 as well as His_6-TRAF3, demonstrating specific binding to caspase-7.

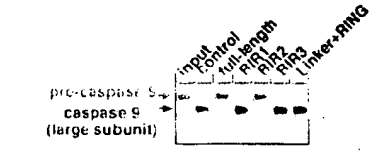

FIG. 4. **BIR2 of XIAP blocks cytochrome *c*-induced processing of pro-caspase-9.** Pro-caspase-9 was *in vitro* translated in the presence of [^{35}S]L-methionine and then added to cytosolic extracts (*input*). Identical aliquots of the ^{35}S-pro-caspase-9/extract mixtures were then incubated with 10 μM cytochrome *c* and 1 mM dATP alone (*control*) or in combination with various GST fusion protein (2 μM) containing the BIR1, BIR2, BIR3, or RING domains of XIAP as indicated at 30 °C for 30 min. Reactions were stopped by boiling in an equal volume of Laemmli buffer and analyzed by SDS-PAGE/autoradiography. The positions of the unprocessed pro-caspase-9 and processed large subunit of caspase-9 are indicated.

been obtained to date indicating that XIAP cleavage by caspases occurs under physiological circumstances in intact cells. The specificity of the *in vitro* binding results was confirmed by experiments in which other His_6-tagged, recombinant proteins, including caspase-6, caspase-8, and TRAF-3 were tested and found not to bind the BIR2-containing fragment of XIAP (Fig. 3*B*).

Recently, it has been demonstrated that release of cytochrome *c* from mitochondria occurs during apoptosis and results in caspase activation by inducing formation of a protein complex containing Apaf-1 and pro-caspase-9 (reviewed in Ref. 21). Moreover, we have found that XIAP, cIAP-1, and cIAP-2 can bind and inhibit pro-caspase-9, preventing it from becoming processed when exposed to cytochrome *c* and Apaf-1 (24). We therefore tested whether BIR2 was sufficient for preventing cytochrome *c*-induced processing of pro-caspase-9 in cytosolic extracts. As shown in Fig. 4, full-length XIAP and the BIR2-containing fragment of XIAP completely blocked cytochrome *c*-induced processing of ^{35}S-labeled *in vitro* translated pro-caspase-9 in cytosolic extracts, whereas BIR1, BIR3, and RING-containing fragments of XIAP were ineffective. Thus, the BIR2 domain of XIAP appears to be sufficient for blocking the

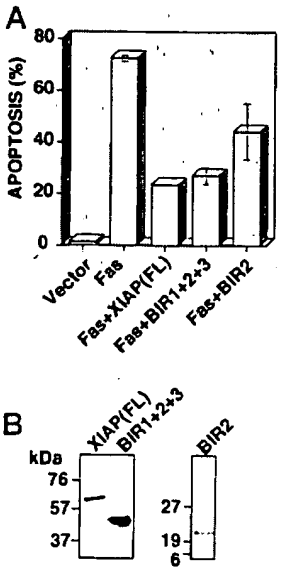

FIG. 5. **BIR2 of XIAP blocks Fas-induced apoptosis.** 293 cells were transiently transfected with 0.5 μg of pEGFP (*Vector*), 1 μg of pcDNA control plasmid, or 1 μg of pcDNA-Fas plasmid together with either 5 μg of pcDNA3 or pcDNA3-myc-XIAP (full-length), myc-XIAP (BIR1+2+3), or myc-XIAP (BIR2). One day later, cells were recovered, and the percentage displaying apoptotic morphology was determined by DAPI staining (mean ± S.E.; $n = 3$) among GFP positive cells (*A*), or lysates were prepared for immunoblot analysis of 25 μg of total protein using anti-Myc antibody and an emission chemiluminescence-based detection method (*B*).

earliest step in the apoptotic pathway triggered by cytochrome *c*, *i.e.* processing of pro-caspase-9.

The effects of the BIR2 portion of XIAP on apoptosis in cells were examined by expressing this or other regions of XIAP in 293 cells, which were induced to undergo apoptosis by overexpression of Fas. Transient transfection of 293 cells typically resulted in over half the cells becoming apoptotic, as determined by DAPI staining and UV microscopic analysis (Fig. 5*A*). In contrast, co-expressing Fas with plasmids producing Myc-tagged full-length XIAP, BIR1+2+3, or BIR2 alone significantly reduced the percentage of transfected cells undergoing Fas-induced apoptosis. Although the BIR2 fragment was less potent at suppressing Fas-induced apoptosis than full-length XIAP or the BIR1+2+3 fragment of XIAP, it also may have been produced at lower levels in 293 cells (Fig. 5*B*). In contrast, co-expression of Fas with plasmids producing Myc-tagged fragments of XIAP that lacked BIR2, including myc-BIR1 and myc-BIR1+3, failed to suppress apoptosis (not shown). These BIR2-lacking XIAP mutants however were produced at levels comparable with or greater than those of myc-BIR2, based on control transfections experiments using 293 cells that were not induced to undergo apoptosis by Fas (data not presented). Taken together, these findings support the idea that the BIR2 region of XIAP is necessary and sufficient for inhibiting caspases and cellular apoptosis.

Although most IAP family proteins contain two or three copies of the BIR domain, the data provided here suggest that not all BIRs are equivalent in their ability to bind and inhibit caspases. In XIAP, it appears that only the second BIR domain is capable of inhibiting active caspases-3 and -7. Despite their extensive amino acid sequence similarity to BIR2, the BIR1 and BIR3 domains did not inhibit these caspases. Although the issue of protein folding when dealing with mutants is always a potential concern, these findings nevertheless suggest that different BIR domains within IAP family protein may play unique roles in the suppression of cell death. An intriguing idea is that different BIRs may target different subsets of caspases, although no direct evidence for this is yet available. Alternatively, it has been suggested that BIR domains represent a novel type of protein interaction domain. Miller and colleague have noted interactions of the baculovirus IAP family proteins with the *Drosophila* cell death proteins *reaper* and *doom* (22, 23), neither of which are caspases. Efforts to identify the targets of each of the individual BIR domains within IAP family proteins and to understand the structural basis for their differential inhibition of caspases will provide additional insights into the mechanisms by which IAPs suppress apoptosis.

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